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# The separation of glutathione transferase subunits by using reverse-phase high-pressure liquid chromatography

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A simple method is described for the separation and quantification of the subunits of GSH transferases present in rat tissue extracts. This method, involving GSH-agarose affinity chromatography followed by reverse-phase h.p.l.c., is rapid and sufficiently sensitive to measure 5 $\mu$g of each subunit in a mixture. Examples are given of its application to extracts of rat kidney, adrenal, testicular interstitial cells and seminiferous tubules. The analysis of seminiferous tubules indicates that the technique may be of value for the identification of novel subunits. Preliminary separations of subunits from human GSH transferases are also described.

## INTRODUCTION

Soluble glutathione (GSH) transferases (EC 2.5.1.18) are enzymes associated with the detoxication of electrophiles (Mannervik, 1985; Ketterer *et al.*, 1986), the reduction of fatty acid hydroperoxides and pyrimidine hydroperoxides (Tan *et al.*, 1986), the isomerization of prostaglandins (Christ-Hazelhof & Nutgeren, 1979) and non-covalent binding affinity for a range of compounds with hydrophobic moieties (Ketterer *et al.*, 1978).

GSH transferases appear to be widespread in both plant and animal kingdoms and have been studied in some detail in rat and man. In each species examined, GSH transferases have proved to be dimers of a number of subunits with $M_r$ values in the region of 25000 (Mannervik, 1985; Ketterer *et al.*, 1986). Each subunit has a characteristic enzymic activity, and in both homo- and hetero-dimers the activity of each subunit is expressed independently of the other (Danielson & Mannervik, 1985).

The present paper describes a method of value for the study of tissue samples for their content of GSH transferase subunits. This method, which is rapid and relatively sensitive, has been applied to GSH transferases in the rat, and may also be of value in studies of human tissue.

## MATERIALS AND METHODS

### Purified enzymes

Purified GSH transferases used to calibrate the system were isolated from liver, kidney or testis of Wistar-strain rats inbred at the Middlesex Hospital Medical School and from human liver and placenta made available by King's College Hospital.

Rat GSH transferases 1–1, 2–2, 3–3, 4–4 and 7–7 were purified by the procedures of Beale *et al.* (1983) and Meyer *et al.* (1985). GSH transferase 6–6 was purified from rat testis by a modification of the method of Beale *et al.* (1983) in which a fraction of isoelectric point pH 5.8 was further purified on hydroxyapatite. The preparation moved as a single band on SDS/polyacrylamide-gel electrophoresis with a mobility slightly greater than that of subunits 3 and 4 (Jakoby *et al.*, 1984), had a similar tryptic peptide map to GSH transferases 3–3 and 4–4 and the same *N*-terminal sequence as reported for subunit 4 by Mannervik *et al.* (1985).

Human GSH transferases of the $\alpha$ class (Mannervik *et al.*, 1985) and GSH transferase $\mu$ were from an individual human liver and were separated as follows. A combined GSH transferase fraction, prepared by affinity chromatography in accordance with Vander Jagt *et al.* (1985), was separated into GSH transferase $\mu$ and a peak containing GSH transferases of the $\alpha$ class by the method of Hussey *et al.* (1986). GSH transferases of the $\alpha$ class were further fractionated by chromatofocusing in the pH range 9 to 6 using a fast protein liquid-chromatography system (Pharmacia, Uppsala, Sweden). This method gives at least 16 components grouped into six major fractions eluted at pH 8.15, 8.05, 7.80, 7.60, 7.35 and 7.15. GSH transferase $\pi$ was isolated from a human placenta by obtaining a combined GSH transferase fraction by affinity chromatography (Vander Jagt *et al.*, 1985) and separating this fraction by anion-exchange fast protein liquid chromatography in a Mono-Q column (Pharmacia, Uppsala, Sweden) buffered with 10 mM-Bistris/HCl at pH 6.1 made 0.5 mM with respect to dithiothreitol and 1 mM with respect to GSH. The column was eluted with a KCl gradient, and 90% of the protein was eluted as a single peak at 0.1 M-KCl. The enzymic activities of this peak were comparable with, and the *N*-terminal sequence identical with, those of GSH transferase $\pi$, as isolated and described by Guthenberg *et al.* (1985).

Purified GSH transferases from all sources migrated as single bands on SDS/polyacrylamide-gel electrophoresis.

### Tissue samples for analysis

Tissue samples were prepared for analysis by using a small-scale version of the method of Vander Jagt *et al.* (1985) as follows. A soluble supernatant fraction was

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obtained from tissues homogenized in 10 mM-potassium phosphate buffer, pH 7.0, made 0.15 M with respect to KCl (KCl/phosphate buffer). The soluble supernatant was passed through a 1.2 cm × 1.6 cm GSH-agarose column equilibrated with KCl/phosphate buffer. After the column had been washed with 3.5 ml of KCl/phosphate buffer, the GSH transferase fraction was eluted with 6 ml of 50 mM-Tris containing 10 mM-GSH and adjusted to pH 9.0 with KOH at room temperature. The first 1 ml of eluate was discarded and the next 3 ml was retained for h.p.l.c. The temperature was maintained at 4 °C throughout. The affinity column was regenerated by washing with 6 M-guanidinium chloride, pH 7.5, and re-equilibration with KCl/phosphate buffer.

Testicular interstitial cells (steroidogenic tissue) were separated from seminiferous tubules (spermatogenic tissue) by dissection in accordance with Hall *et al.* (1969).

**Analysis of subunits by reverse-phase h.p.l.c.**

H.p.l.c. analysis of GSH transferases was carried out on a 10 cm × 0.8 cm Waters $\mu$Bondapak $C_{18}$ reverse-phase column in a Z module with a Waters system (Milford, MA, USA). The solvents were water (solvent A) and 0.1% (v/v) trifluoroacetic acid in acetonitrile (solvent B). In the case of rat tissues the sample was injected at 35% (v/v) solvent B and a linear gradient run from 35% to 55% (v/v) solvent B over 60 min with a flow rate of 1.5 ml/min. Polypeptides were detected at 214 nm. Where human tissues were analysed, a shallower gradient from 40% to 50% (v/v) solvent B proved necessary in order to separate subunits from GSH transferases $\mu$ and $\pi$.

There was evidence for a memory effect at very high sample loadings. As a precaution blank runs were occasionally introduced during a long series of analytical runs.

In the first instance, it was assumed that only GSH transferases were collected from the affinity column and that the identity of the polypeptide peaks separated by h.p.l.c could be determined by reference to the retention times of subunits from purified GSH transferases. In questions of doubt, or when apparently novel peaks were observed, peak fractions were further investigated by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970), h.p.l.c. analysis of tryptic peptides and *N*-terminal amino acid sequencing with an Applied Biosystems (Foster City, CA, U.S.A.) 470A protein sequencer.

The separation of subunits by h.p.l.c. was quantified from the peak area expressed as $A_{214}$ × ml, as obtained from the recorder. In order to convert peak area into protein content, the $\epsilon_{214}$ for each subunit was obtained by multiplying its $\epsilon_{280}$, calculated from its known tyrosine and tryptophan content (Pickett *et al.*, 1984; Lai *et al.*, 1984; Telakowski-Hopkins *et al.*, 1985; Ding *et al.*, 1985; Suguoka *et al.*, 1985; Rothkopf *et al.*, 1986), by the ratio of $A_{214}$ to $A_{280}$ obtained from its absorption spectrum. The $\epsilon_{214}$ values for subunits 1, 2, 3, 4 and 7 were $21 \times 10^4$, $36 \times 10^4$, $42 \times 10^4$, $34 \times 10^4$ and $35 \times 10^4$ $M^{-1} \cdot cm^{-1}$ respectively.

## RESULTS

### Separation

Fig. 1 shows subunits from purified rat GSH transferases 1–1, 2–2, 3–3, 4–4 and 7–7 eluted from h.p.l.c. in the order 3, 4, 7, 2 and 1 with retention times 25, 28,

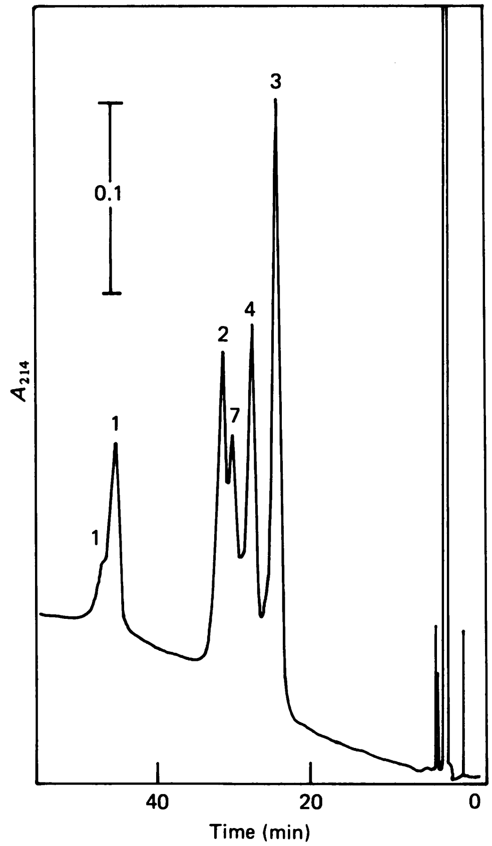


**Fig. 1. Separation of rat GSH transferase subunits by reverse-phase h.p.l.c.**

A mixture of purified GSH transferase homodimers 1–1, 2–2, 3–3, 4–4 and 7–7 in 0.1 M-sodium phosphate buffer, pH 6.8, was applied to a $\mu$Bondapak $C_{18}$ column and eluted with a gradient of solvent B (0.1% trifluoroacetic acid in acetonitrile) in solvent A (water) as described in the text. Numbers refer to GSH transferase subunits.

30, 32 and 46 min respectively. Subunit 1 separated into two major peaks, each of which, when re-run, was eluted as a single peak with its original retention time. It was not possible to distinguish between these two components by h.p.l.c. analysis of tryptic peptides; however, it has been reported that subunit 1 is heterogeneous (Beale *et al.*, 1982; Lai *et al.*, 1984; Pemble *et al.*, 1986), and it is possible that h.p.l.c. provides a means of separating at least two different forms. It has been possible to recover enzymic activity from the subunit 2 peak by freeze-drying. Means of restoring activity to other fractions have yet to be discovered.

Fig. 2 shows the elution of GSH transferase 6–6 isolated from testis. It would appear that it has been misnamed as a homodimer, since approximately equal quantities of two components are obtained, one with a retention time of 28 min, which is similar to that of subunit 4, and another with a retention time of 37 min and therefore well resolved from both subunits 1 and 2. As a result of this analysis this GSH transferase preparation is referred to below as GSH transferase '6–6'.

Figs. 3(*a*) and 3(*b*) show the application of this technique to soluble supernatant fractions from rat liver and kidney respectively. In the liver, subunits 1, 2, 3 and

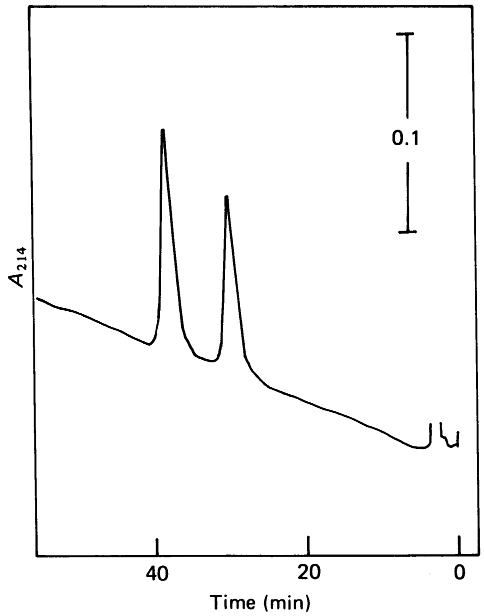


**Fig. 2. Resolution of GSH transferase '6–6' into two components by reverse-phase h.p.l.c.**

GSH transferase '6–6' was purified from testis and applied to reverse-phase h.p.l.c. as described in the text.

4 are clearly identified and all four subunits are abundant. On the other hand, in the kidney, subunits 3 and 4 are only minor components, subunit 7 (not seen in normal liver) and subunit 2 are major components, and subunit 1 is the most abundant of all (see Fig. 3*b*). In liver a small peak with a retention time of approx. 37 min and one or two small peaks eluted after the main components of subunit 1 are often observed. The former peak may represent the more slowly eluted subunit of GSH transferase '6–6', since GSH transferase 4–6 has been observed in liver in small amounts (see Hayes, 1984; Ketterer *et al.*, 1986) and the latter peaks may be variants of subunit 1.

Figs. 4(*a*) and 4(*b*) show the application of this technique to seminiferous tubules and testicular interstitial cells, and Figs. (5*a*) and 5(*b*) to adrenals of male and female rats. The GSH transferase fraction of seminiferous tubules separates into five peaks. The first and third are subunits 3 and 2 respectively. The second peak is shown, on SDS/polyacrylamide-gel electrophoresis, to consist of both subunit 4 and the more rapidly eluted component of GSH transferase '6–6'. The fourth peak consists of the more slowly eluted component of GSH transferase '6–6', and the fifth peak has an elution volume less than that of subunit 1 and a mobility on SDS/polyacrylamide-gel electrophoresis similar to those of subunits 3 and 4. Further work is required to determine its identity.

The GSH transferase fraction of the testicular interstitial cells is resolved into subunits 3, 4, 2 and a small amount of 7. The simplest analysis so far is the adrenal gland (Fig. 5), which contains predominantly subunit 2. The adrenal of the male rat is shown to contain more GSH transferase than that of the female.

The h.p.l.c. of a mixture of purified human GSH transferases is shown in Fig. 6. GSH transferases $\pi$ and $\mu$ give rise to single subunit peaks at 34 and 37 min,

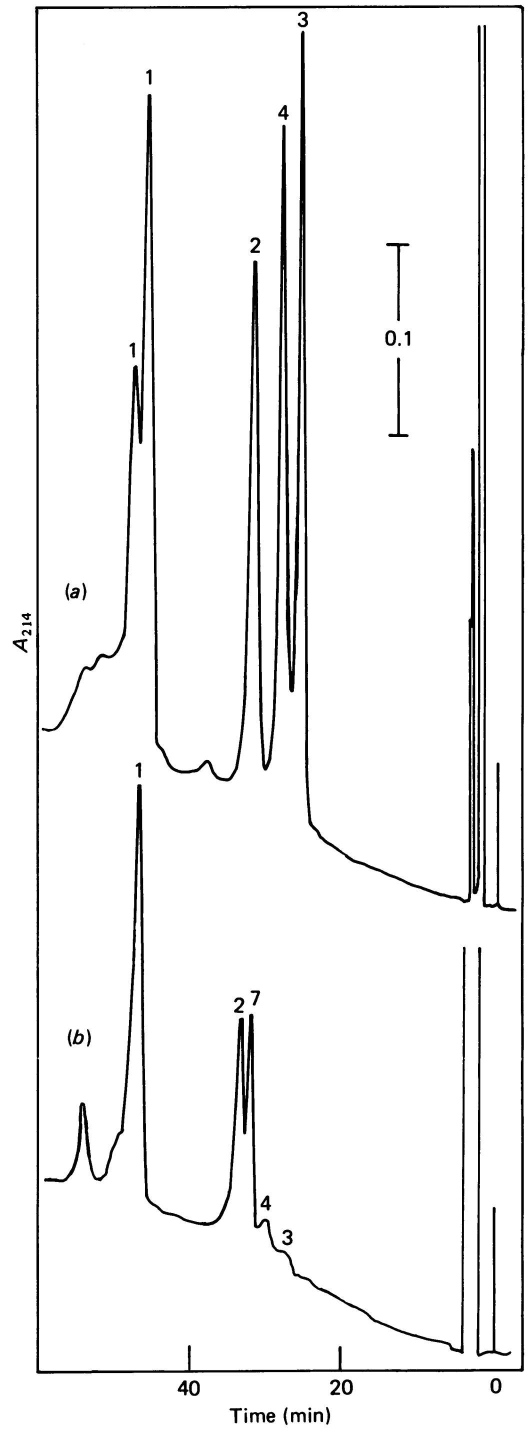


**Fig. 3. Comparison of the GSH transferase subunit compositions of rat liver and kidney**

The GSH transferase fraction was isolated from kidney and from liver by affinity chromatography as described in the text. The GSH transferase fraction equivalent to 0.15 g of liver (*a*) and that equivalent to 0.24 g of kidney (*b*) were analysed by reverse-phase h.p.l.c. as described in the text. Numbers refer to GSH transferase subunits.

whereas GSH transferases of the $\alpha$ class give two asymmetric peaks, referred to here as $\alpha_x$ and $\alpha_y$, with retention times of 48 and 56 min respectively. When six major GSH transferase fractions of the $\alpha$ class were separated by chromatofocusing, as described in the

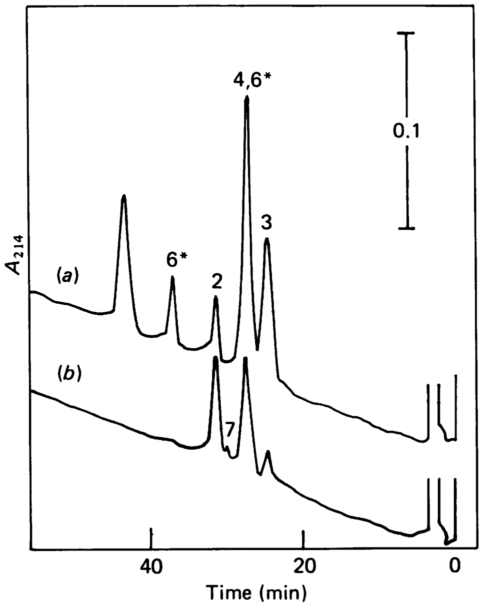


**Fig. 4. Comparison of GSH transferase subunit compositions of seminiferous tubules and interstitial cells of rat testis**

Seminiferous tubules and interstitial cells of rat testis were separated by dissection and the GSH transferase fraction was obtained from each by affinity chromatography as described in the text. The GSH transferase fractions of seminiferous tubules (*a*) and interstitial cells (*b*) equivalent to 0.4 g testis were analysed by reverse-phase h.p.l.c. as described in the text. Numbers refer to GSH transferase subunits.

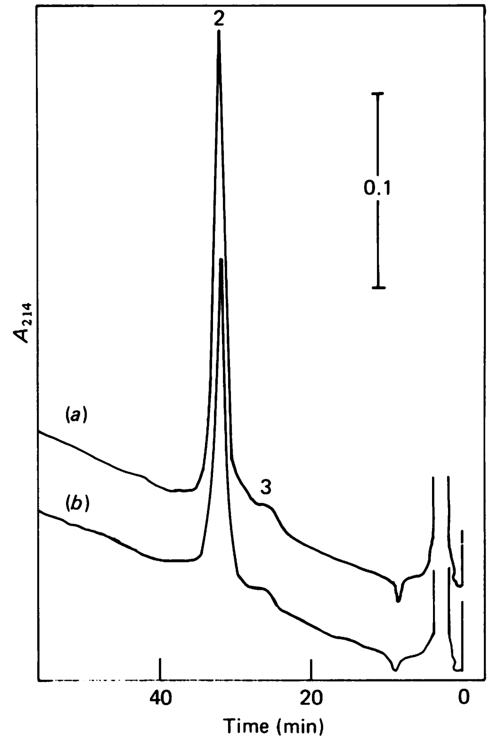


**Fig. 5. Comparison of the GSH transferase subunit compositions of adrenal glands of male and female rats**

The GSH transferase fraction was isolated from rat adrenal glands by affinity chromatography as described in the text. The GSH transferase fractions equivalent to 0.5 g of adrenal glands of male (*a*) and female (*b*) rats were analysed by reverse-phase h.p.l.c. as described in the text. Numbers refer to GSH transferase subunits.

Materials and methods section, the most basic, eluted at pH 8.5, gave solely $\alpha_x$ on h.p.l.c., the least basic, eluted at pH 7.15, gave solely $\alpha_y$ and the four intermediate fractions gave both $\alpha_x$ and $\alpha_y$ in approximately equal amounts. Since peaks $\alpha_x$ and $\alpha_y$ are asymmetric on h.p.l.c., they may contain more than one component. The existence of several forms of $\alpha_x$ and $\alpha_y$ subunits would explain the existence of what appears to be at least four $\alpha_x\alpha_y$ heterodimers.

Apart from the coincidence of the more rapidly eluted subunit from GSH transferase '6–6' and subunit 4, all the peaks separated above run as single bands on SDS/polyacrylamide-gel electrophoresis and appear to be pure.

### Quantification

During the initial separation on the affinity column 1–3% of the total GSH transferase activity (with 1-chloro-2,4-dinitrobenzene as a substrate) passed through the GSH–agarose column unretained, approx. 95% was eluted by 50 mM-Tris containing 10 mM-GSH, and about 2% remained strongly bound to the matrix. Of the activity eluted by Tris/GSH, 90% (i.e. 85% of the total activity) was eluted in the 2nd–4th-ml region of eluate. This was collected for analysis by h.p.l.c.

On h.p.l.c. a linear relationship existed between the amount of each subunit applied and the amount recovered in the range 5–150 $\mu$g. However, the percentage recovered varied with the subunit, being 75, 75, 65, 40 and 35% for subunits 1, 2, 3, 4 and 7 respectively.

The usefulness of the quantification can be demonstrated by the following results obtained from the analysis of an 80 mg sample of rat liver, where 125, 40, 28 and 30 $\mu$g of subunits 1, 2, 3 and 4 respectively were recovered from the h.p.l.c. With corrections to account for losses on both affinity chromatography and h.p.l.c., it is estimated that the soluble supernatant fraction from 80 mg rat liver contained 196, 63, 51 and 88 $\mu$g of subunits 1, 2, 3 and 4 respectively.

These results compare well with estimates obtained by immunochemical procedures. Well-established immunochemical measurements relate to ligandin, which is a mixture of GSH transferases 1–1 and 1–2. The ligandin content of liver cytosol, based on the calculation that the cytosol is 60% the hepatocyte volume (Drabkin, 1975), is 0.1 mM (Tipping & Ketterer, 1981). From the present method 80 mg of liver contained 259 $\mu$g of subunit 1 and 2. Thus their concentration in the calculated cytosol volume, namely 48 $\mu$l, is also 0.1 mM.

## DISCUSSION

Analysis of tissue extracts for GSH transferases have involved either direct immunochemical analysis (Bass *et al.*, 1977) or the separation of isoenzymes and their identification and quantification by enzymic activity (Ketterer *et al.*, 1985, 1986). Immunochemical analysis requires the raising of antibodies that discriminate between highly homologous proteins, and so far a range of monospecific antibodies capable of distinguishing all known subunits has not been described (see Hayes &

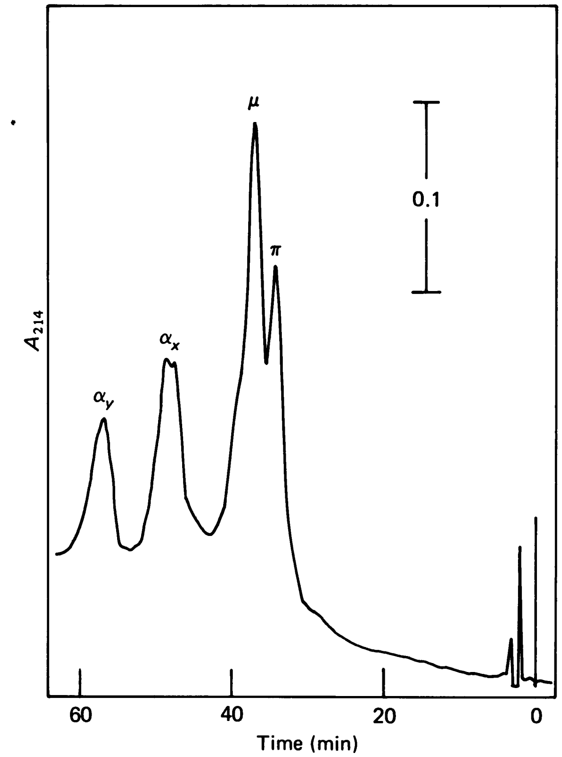


**Fig. 6. Separation of human GSH transferases by reverse-phase h.p.l.c.**

GSH transferase homodimers purified from human tissue by affinity chromatography, absorption chromatography and chromatofocusing were analysed by reverse-phase h.p.l.c. as described in the text. Subunits from GSH transferases $\pi$ and $\mu$ gave single peaks; subunits of the $\alpha$ class of GSH transferases gave two peaks referred to as $\alpha_x$ and $\alpha_y$, in which $\alpha_x$ is obtained from the most basic homodimer and $\alpha_y$ the least basic.

Mantle, 1986). The separation of isoenzymes and their quantification by enzymic activity as previously published are far from ideal. It is difficult to estimate all the losses that occur in the various steps involved in procedures designed to separate all homodimers and heterodimers. In addition, there are variations in the specific enzymic activities of the various isoenzymes from one laboratory to another, which introduces uncertainty into content as expressed as weight or moles of enzyme. Even when an analysis of a tissue has been made by this method, the potential of a tissue to detoxify known substrates for GSH transferases requires the conversion of an analysis expressed as homodimers and heterodimers into one expressed as subunits, since the subunits are kinetically independent.

In the present method subunits, not dimers, in a tissue extract are separated and estimated as protein, not enzymic, activity. Results are obtained that are quickly interpreted. Because only two steps are involved, losses are readily monitored, and quantification appears to be good. In support of this is the observation that results for subunits 1 and 2 in rat liver obtained by the present method are comparable with those obtained by immunochemical analysis (Tipping & Ketterer, 1981).

This method does not resolve all mixtures of GSH transferases; however, subunits 1, 2, 3, 4 and 7 separate well and therefore those tissues that contain these subunits as major components are readily analysed. This is illustrated here by analyses of liver, kidney, adrenal and testicular interstitial cells. When an attempt is made to analyse GSH transferases of seminiferous tubules, resolution is incomplete, since subunit 4 and the faster eluted subunit 6 are inseparable. The analysis can be completed by an additional SDS/polyacrylamide-gel-electrophoresis separation. In addition, a novel peak is observed, which requires further study to determine whether or not it is a GSH transferase subunit.

This technique may be applicable to GSH transferases from other species. Promising results have been obtained with human GSH transferases. The clear-cut separation of two fractions of human GSH transferase subunits from the $\alpha$ class is of considerable interest.

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